EXHIBIT A

Docket 7/30/02
MWH

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/749,233	12/27/2000	Theodorus Cornelis Schaap	1/98407 US/D1	5760

7590 04/30/2002

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EXAMINER	
BASKAR, PADMAVATHI	

ART UNIT	PAPER NUMBER
1645	10

DATE MAILED: 04/30/2002

Please find below and/or attached an Office communication concerning this application or proceeding.

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PTO-90C (Rev. 07-01)

Applicant's Copy

Office Action Summary	Application No. 09/749,233	Applicant(s) SCHAAP ET AL.
	Examiner Padmavathi v Baskar	Art Unit 1645

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *22 March 2002* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *7-22 and 24-29* is/are pending in the application.

4a) Of the above claim(s) *9-22 and 24-29* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *7 and 8* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *7-22 and 24-29* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) 5 .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other:

DETAILED ACTION

1. Applicant's amendment filed on (3/22/02) in Paper # 9 is acknowledged. Claims 7-22 and 24-29 are pending in the application.

Election

2. Applicant's election of Group I claims 7-8 with respect to SEQ.ID.NO: 3 (3/22/02) in Paper # 9 is acknowledged. The Office notes that the election of SEQ.ID.NO: 3 is the election of an invention under restriction requirement made in Paper # 9. Applicant requests the examiner to include claims 16-20 in the elected invention. Claims 16-20 are directed to a vaccine composition and are viewed as a separate invention since the vaccine composition comprises polypeptide or recombinant DNA molecule or live recombinant carrier and an additional immunogen of a poultry pathogen in a suitable adjuvant to induce protective immunity to Eimeria. A reference, which would anticipate the invention of group I, would not necessarily anticipate or make obvious the invention of group III, vaccine composition since the composition not only consists of peptide but also other components such as additional immunogen of a poultry pathogen and adjuvant. Burden in examining materially different groups having materially different issues such as enablement exist. Therefore, claims 9-22 and 24-29 are withdrawn from consideration.

Information Disclosure Statement

3. Information Disclosure Statement filed on 12/27/00 (Paper # 5) is acknowledged and a signed copy is attached to this Office action.

Priority

4. Applicant claims domestic priority under 35 U.S.C. 120 based upon a previously filed 09/411,578 application, now U.S.Patent 6,203,801. This should appear as the first sentence of

the specification following the title, preferably as a separate paragraph.

Specification - Informalities

5. Claims shoud begin with "I claim" or ""We claim" or "What is claimed is" on page 43.

Claim Rejections - 35 USC § 101

6. 35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefore, subject to the conditions and requirements of this title.

7. Claims 7 and 8 are rejected under 35 U.S.C. § 101 because the claimed invention is directed to non-statutory subject matter. The product, hydrophilic polypeptide as claimed, has the same characteristics as that found in nature. To overcome this rejection the Examiner suggests the amendment of the claims to include purity limitations, which would distinguish the characteristics of applicant's product from the product as it exists in nature. It is further suggested that such limitation include the terminology " purified and isolated" (i.e. if such purity is supported in the specification) and/or a description of what applicant's protein is "free of" relative to the natural source. (see Farbenfabriken of Elberfeld Co. v. Kuehmsted, 171 Fed. 887, 890 (N.D. Ill. 1909) (text of claim at 889); Parke-Davis & Co. v. H.D. Mulford Co., 189 Fed. 95, 103, 106, 965 (S.D.N.Y. 1911) (claim 1); and In re Bergstrom, 427 F.2d 1394, 1398, 1401-1402 (CCPA 1970).

Claim Rejections - 35 USC 112, first paragraph

8. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to

make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

9. Claims 7-8 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for an isolated polypeptide of Eimeria or E. tenella comprising the amino acid sequence SEQ.ID.NO: 3 does not reasonably provide enablement for an isolated polypeptide of Eimeria or E. tenella comprising an amino acid sequence that shares at least 70% sequence homology with SEQ.ID.NO: 3. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Scope of enablement requires that the specification teach those in the art how to make and use the invention commensurate with the scope of the claimed invention without undue experimentation and includes an analysis of: (1) the nature of the invention, (2) the state of the prior art, (3) the predictability or lack thereof in the art, (4) the amount of direction or guidance present, (5) the presence or absence of working examples, (6) the quantity of experimentation necessary, (7) the relative skill of those in the art, and (8) the breadth of the claims.

With regard to %identity, the specification is not enabled for a polypeptide comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO: 3 because it is unclear to one skilled in the art what sequences are embraced by the claim and what point mutation, deletions, insertions and rearrangements have been done to an isolated peptide which would result in a polypeptide comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO 3. If it is unclear to one skilled in the art what sequences are embraced by a claim which is based on a specification to determine percent homology which would give rise to an isolated polypeptide which has 70% sequence homology with SEQ.ID.NO

3, the specification is non-enabling, since one skilled in the art would not be able to make and use those sequences without undue experimentation.

The specification provides guidance and direction with regard to an isolated hydrophilic polypeptide comprising an amino acid sequence as set forth in the SEQ.ID.NO: 3 (example 1 and 2) which is designated as peroxidoxin-like polypeptide. However, Applicant has not set forth which amino acid (s) can be deleted or inserted or substituted in the polypeptide (SEQ.ID.NO 3) to give rise to a polypeptide comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO 3. After these alterations or modifications whether the polypeptide can still retain the activity as presently claimed is not set forth clearly in the specification.

It is well known that for proteins, for example, even a single amino acid change can destroy the function of the biomolecule. The amino acid sequence of a protein determines its structural and functional properties, predictability of which changes can be tolerated in a protein's amino acid sequence and still retain similar activity requires a knowledge of and guidance with regard to which amino acids in the protein's sequence, if any, are tolerant of modification and which are conserved (i.e. expected intolerant to modification), and detailed knowledge of the ways in which the proteins' structure relates to its function. However, the problem of predicting protein structure from mere sequence data of a single protein (70% homology) and in turn utilizing predicted structural determinations to ascertain functional aspects of the protein and finally what changes can be tolerated with respect thereto is extremely complex (Bowie et al. Science, Vol. 247: 1990; p. 1306; p. 1308) and is well outside the realm of routine experimentation.

While recombinant and mutagenesis techniques are known, it is not routine in the art to screen for multiple substitutions or multiple modifications of other types and the positions within

the protein's sequence where amino acid modifications can be made with a reasonable expectation of success in obtaining similar activity are limited in protein and the result of such modifications is unpredictable based on the instant disclosure.

With regard to function of a polypeptide comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO 3, Houghten et al. (Vaccines, 1986, Edited by Fred Brown: Cold Spring Harbor Laboratory) teach that changes/modifications (addition, substitution, deletion or inversion) of one or more amino acids in a polypeptide will alter antigenic determinants and therefore affect antibody production (p. 21) as well as antibody binding. Houghten et al. also teach that "... combined effects of multiple changes in an antigenic determinant could result in a loss of [immunological] protection." and "A protein having multiple antigenic sites, multiple point mutations, or accumulated point mutations at key residues could create a new antigen that is precipitously or progressively unrecognizable by any of the antibodies..." (p. 24). Houghten et al. teach that point mutations at one key antigen residue could eliminate the ability of an antibody to recognize this altered antigen (p. 24). It is not always possible to make the variants that retain immunodominant regions and immunological activity if the regions have been altered. Therefore, a polypeptide comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO: 3 would result in a peptide without any function.

Thus, applicants have not provided sufficient guidance to enable one of ordinary skill in the art to make and use the claimed polypeptide in a manner reasonably correlated with the scope of the claims. The scope of the claims must bear a reasonable correlation with the scope of enablement (In re Fisher, 166 USPQ 19 24 (CCPA 1970)). Without such guidance, the changes made in the protein to obtain a polypeptide comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO 3 renders activity unpredictable and the

experimentation left to those skilled in the art is unnecessarily, and improperly, extensive and undue.

Claim Rejections - 35 USC 102

10. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

11. Claims 7-8 are rejected under 35 U.S.C. 102(b) as being anticipated by Karkhanis et al 1991 (Infection and Immunity, 59; 983-989).

Claims are directed to the polypeptide of Eimeria or a polypeptide of E.tenella comprising an amino acid sequence that shares at least 70% homology with SEQ.ID.NO: 3.

Karkhanis et al 1991 disclose E. tenella polypeptides prepared from sporulated oocysts and sporozoites. Sporulated oocysts and sporozoites were sonicated in PBS. The sonicated material was supplemented with 0.1% Zwittergent and extracted for 18 hours. Following centrifugation the supernatant was subjected to gel filtration, which yielded 26kD and 22 kD polypeptides (see abstract, page 983, right column and figure 11 and 12). The claimed hydrophilic polypeptides were present in the supernatant of extracts obtained from E.tenella sporulated oocysts and sporozoites of Karkhanis et al. The supernatant (hydrophilic portion) of the extract was soluble in PBS and hence contains hydrophilic polypeptides of E. tenella.

Applicant's use of the open-ended term "comprising "in claims 7-8 fails to exclude unrecited steps or ingredients and leaves the claims open for inclusion of unspecified ingredients, even in major amounts. Therefore, the claims read on the supernatant of the

extracts of E.tenella oocysts and sporozoites which inherently comprises the hydrophilic polypeptides that comprise an amino acid sequence as set forth in the SEQ.ID.NO: 3. Thus the prior art anticipated the claimed invention.

In the absence of evidence to the contrary the disclosed prior art polypeptide and the claimed polypeptide are the same. Since the Office does not have the facilities for examining and comparing applicants' claimed polypeptide with the polypeptide of the prior art, the burden is on applicant to show a novel or unobvious difference between the claimed product and the product of the prior art. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and In re Fitzgerald et al., 205 USPQ 594.

Status of Claims

12. No claims are allowed.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Padma Baskar whose telephone number is (703) 308-8886. The examiner can normally be reached on Monday through Friday from 6:30 AM to 4 PM EST

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lynette Smith can be reached on (703) 308-3909. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-1235.

Padma Baskar Ph.D

4/10/02

LYNETTE R. F. SMITH
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

EXHIBIT B

Notice of Allowability	Application No. 09/749,233	Applicant(s) SCHAAP ET AL.
	Examiner Padmavathi v Baskar	Art Unit 1645

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *7/29/03 and 8/14/03*.
2. ☒ The allowed claim(s) is/are *7-8 have been entered as 1-2 respectively*.
3. ☐ The drawings filed on _____ are accepted by the Examiner.
4. ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☒ All b) ☐ Some* c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☒ Certified copies of the priority documents have been received in Application No. *09/411,578*.
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.
5. ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
   (a) ☐ The translation of the foreign language provisional application has been received.
6. ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application. **THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

7. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

8. ☐ CORRECTED DRAWINGS must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
   1) ☐ hereto or 2) ☐ to Paper No. ____.
   (b) ☐ including changes required by the proposed drawing correction filed _____, which has been approved by the Examiner.
   (c) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No. _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet.

9. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1☐ Notice of References Cited (PTO-892)
2☐ Notice of Informal Patent Application (PTO-152)
3☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
4☒ Interview Summary (PTO-413), Paper No. *19,21*.
5☐ Information Disclosure Statements (PTO-1449), Paper No. ____.
6☒ Examiner's Amendment/Comment
7☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
8☐ Examiner's Statement of Reasons for Allowance
9☐ Other

Vb

LYNETTE R. F. SMITH
SUPERVISORY PATENT
TECHNOLOGY CENTER

Examiner's Amendment

1. The amendment filed on 7/29/03 (paper # 18) and the supplemental amendment filed on 8/14/03 (paper # 20) has been entered. Claims 7-8 have been amended. Claims 1-6 and 9-35 have been canceled. Claims 7-8 are pending in the application.

2, An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Mr. Mark Milstead (see paper # 21).

The application has been amended as follows:

Claim 7, line 2, deleted " an" and inserted therefor -- the –

Claim 8, line 4, after of inserted - - the --

3. In view of the amendment to the claims 7-8, the examiner has withdrawn the rejection of record under 35 U.S.C. 112, second paragraph.

4. In view of the amendments to the claims 7-8 and arguments of record (paper # the examiner has withdrawn the rejection under 35 U.S.C. 102 (b) as being anticipated by Karkhanis et al 1991.

5. Reasons for allowance:

Claims 7-8 are directed to an isolated hydrophilic polypeptide of *Eimera tenella* comprising the amino acid sequence SEQ.ID.NO: 3 and an isolated polypeptide comprising an immunogenic portion of the amino acid sequence of SEQ.ID.NO: 3, said immunogenic portion selectively binds to an anti-Eimera antibody raised against said hydrophilic polypeptide SEQ.ID.NO: 3.

Karkhanis et al 1991 do not disclose an isolated hydrophilic polypeptide of *Eimera tenella* comprising the amino acid sequence SEQ.ID.NO: 3 because the prior art does not specifically disclose the isolated polypeptide containing a conserved part DFTPVCTTE of peroxidoxin molecule. The claimed polypeptide is a novel hydrophilic polypeptide from *Eimera tenella* that is a peroxidoxin like polypeptide in its full- length form comprising 223 amino acids with a calculated molecular weight 25K.D, which is neither disclosed nor suggested by any prior art.

6. Claims 7-8 are allowed and have been renumbered as 1-2 respectively.

7. Any inquiry conceming this communication or earlier communications from the examiner should be directed to Padma Baskar Ph.D. Whose telephone number is (703) 308-8886. The examiner can normally be reached on Monday through Friday from 6:30 A.M. to 4:00 P.M. EST

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lynette Smith can be reached on (703) 308-3909. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-1235.

Padma Baskar Ph.D.

8/19/03

CONCLUSION

Applicants do not believe that any other fee is due in connection with this filing. If, however, Applicants do owe any such fee(s), the Commissioner is hereby authorized to charge the fee(s) to Deposit Account No. 02-2334. In addition, if there is ever any other fee deficiency or overpayment under 37 C.F.R. §1.16 or 1.17 in connection with this patent application, the Commissioner is hereby authorized to charge such deficiency or overpayment to Deposit Account No. 02-2334.

Applicants submit that this application is in condition for allowance, and request that it be allowed. The Examiner is requested to call the Undersigned if any issues arise that can be addressed over the phone to expedite examination of this application.

Respectfully submitted,

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EXHIBIT C

INFECTION AND IMMUNITY, Apr. 1991, p. 1271-1277
0019-9567/91/041271-07$02.00/0

Vol. 59, No. 4

Cross-Protection against Four Species of Chicken Coccidia with a Single Recombinant Antigen

MARK S. J. CRANE,* BAMBI GOGGIN, RONALD M. PELLEGRINO, OWEN J. RAVINO, CHRISTINE LANGE, YASHWANT D. KARKHANIS, KAREN E. KIRK, AND PRASANTA R. CHAKRABORTY

Department of Biochemical Parasitology, Merck, Sharp and Dohme Research Laboratories, P. O. Box 2000, Rahway, New Jersey 07065

Received 4 October 1990/Accepted 9 January 1991

A cDNA clone, SO7′, from an *Eimeria tenella* cDNA library was inserted into the high-expression vector pJC264 and was expressed in *Escherichia coli* as a fusion protein, CheY-SO7′, with a molecular mass of approximately 36 kDa. By using the purified recombinant antigen to immunize young chicks, it was demonstrated that a single dose, without adjuvant, not only protected against severe coccidiosis induced by infection with *E. tenella* but also protected chicks challenged with the heterologous species *Eimeria acervulina*, *E. maxima*, and *E. necatrix*. By using rabbit antiserum raised against recombinant CheY-SO7′, Western blot (immunoblot) analysis of sporulated oocysts of all seven major species of chicken coccidia showed that all species tested contained proteins characteristic of the B class of antigens, of which CheY-SO7′ is representative. It seems likely that a single B antigen could protect chickens against severe coccidiosis caused by infection with any of these *Eimeria* species. Although chicks exposed to prolonged, natural infection develop antibodies to B antigen, active immunization of young chicks with a protective dose of CheY-SO7′ does not elicit a humoral antibody response, suggesting that the partial protection results from cell-mediated effector mechanisms. In addition, the cross-protective nature of the immunity indicates that the response to B antigen is different from that induced by natural infection, which elicits a species-specific immunity. To date, the protection induced by B antigen immunization, although remarkable for a single recombinant protein, is not sufficient to compete with prophylactic chemotherapy.

Coccidiosis, which is caused by obligate, intracellular, protozoan parasites belonging to several species of the genus *Eimeria*, is a major problem for the poultry industry. Currently, the disease, which is controlled by the use of prophylactic chemotherapy, costs the broiler chicken industry worldwide an estimated $250 million per annum (29). Because of the expense of new drug development and the occurrence of drug-resistant parasites, an alternative method of disease control is desirable.

In recent years, modern approaches to vaccine development utilizing hybridoma and recombinant DNA technologies have been applied extensively to the problem of coccidiosis of the domestic chicken (6, 7, 10, 17). However, the recombinant antigens isolated to date either have not been tested in vivo or have been disappointing in their ability to protect broilers against coccidiosis (2, 11, 12, 24, 31). Nevertheless, the development of a subunit (recombinant) vaccine against coccidiosis for the broiler chicken industry still remains an attractive alternative to chemoprophylaxis.

Until recently, it was thought that a live coccidial infection was essential for an effective immune response to protect against a pathogenic challenge (37). There are live coccidiosis vaccines available, such as Immunocox (26) and Coccivac (Sterwin Laboratories), and others are under development for the broiler industry (27, 40). However, recent results from our laboratory and others have shown that live infection is not mandatory for protection but that dead antigen such as a crude parasite extract can also protect against severe coccidiosis (32). These results demonstrated that a dead (subunit) vaccine is feasible and would be economical if protection was induced by a protein which would allow vaccine production by recombinant DNA technology.

In subsequent studies, the crude, protective parasite extract was subfractionated by S200 chromatography, and a protective fraction (fraction V) which contained several polypeptides with a limited molecular weight range was identified (23). By using antisera raised against fraction V and against various other protective parasite extracts, a λgt11 cDNA library of *Eimeria tenella* was screened and clones representing the major polypeptides of fraction V were isolated (35). By using immunopositive clones from the *E. tenella* cDNA library and a novel bacterial expression vector, CheY (18), fusion proteins were engineered, expressed, purified, and tested for protective ability against coccidiosis with broilers in our battery model. Several recombinant proteins from five antigen classes (35) demonstrated partial protection against severe coccidiosis caused by *E. tenella* (8). Moreover, further results demonstrated that one of these recombinants, CheY-SO7′ of the B antigen class, was extremely efficacious and cross-protected against coccidiosis caused by *Eimeria acervulina* and *Eimeria maxima* (8). This report describes further characterization of the fusion protein CheY-SO7′ with respect to its construction, immunogenicity, and protective ability in broiler chicks against coccidiosis caused by four of the seven species of chicken coccidia. In addition, it is demonstrated that class B antigens are present in all seven species of chicken coccidia, indicating that this class of antigen could protect chickens against coccidiosis caused by any of these species.

MATERIALS AND METHODS

Induction and identification of the CheY-SO7′ fusion protein. A cDNA insert with *Eco*RI ends, SO7′ (28), was ligated

* Corresponding author.

to *Eco*RI-linearized pJC264 (18). *Escherichia coli* JM109 was transformed with this recombinant plasmid by following a standard protocol (39). Individual recombinant bacterial colonies were grown in Luria broth containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) to an optical density of 0.6 to 0.7 spectrophotometric units at 600 nm. Induction of the gene was achieved by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co.). Following 18 h of growth after induction, bacteria were harvested by centrifugation, resuspended in sample buffer (0.1 M Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 5% glycerol, 0.001% bromophenol blue) and boiled for 5 min. The total soluble bacterial proteins were resolved in duplicate by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) (25). Following electrophoretic resolution of the proteins, one of the gels was stained with Coomassie blue and the other was processed for immunoblotting (41) and probed with a hyperimmune rabbit antiserum raised against *E. tenella* sporozoites (5).

Purification of recombinant B antigen for vaccination. A pellet of the recombinant bacterial clone CheY-SO7′/9 was disrupted with a French press. The cell lysate was centrifuged (12,000 × *g*, 45 min, 4°C), and the insoluble fusion protein was collected in the pellet. The insoluble recombinant antigen was washed with 1% Triton X-100, which removed a large proportion of pelleted bacterial proteins. The recombinant protein was solubilized in 6 M guanidine HCl–10 mM dithiothreitol at 50°C, diluted further with 7 M urea, and applied to a hydroxyapatite column previously equilibrated with 7 M urea. The column was washed in a stepwise fashion with portions of a 7 M urea solution containing sodium phosphate buffers (pH 6.5) of increasing molarity (10 to 160 mM). Recombinant fusion protein was eluted at 10 and 20 mM sodium phosphate and was stored at −20°C in elution buffer until use. Various modifications of this purification scheme have been used in the preparation of other batches of recombinant B antigen for vaccination studies (22).

Experimental vaccinations. Experimental vaccinations were carried out as described previously (8). Briefly, day-old broiler chicks (Petersen × Arbor Acre) were obtained (Avian Services, Frenchtown, N.J.) and housed in all-stainless-steel, 16-compartment, brooder-type cages (Hazleton Co., Aberdeen, Md.) in rooms where extreme precautions were taken to minimize adventitious exposure to avian coccidia. Experimental antigens diluted in phosphate-buffered saline (PBS, pH 7.4) were administered intramuscularly without adjuvant in the thighs of 2- to 4-day-old broilers. Following immunization, chicks were reared under the conditions described above, with feed and water ad libitum, until three weeks of age. At this time, chicks were transferred to four-compartment, all-stainless-steel poultry batteries (Allentown Caging Equipment Co., Allentown, N.J.), moved to a separate facility, and challenged with a single species of chicken coccidia. Twenty-four hours prior to challenge, feed was removed to ensure that the chicks were hungry. At the time of challenge, pathogenic doses of sporulated oocysts were administered to chicks via feed (15 g of feed per 2.5 ml of water per dose per chick). The inoculated feed was usually fully consumed within 2 h, after which normal feed was restored. The challenge dose, which was determined previously, was sufficient to induce a mean internal coccidial lesion score (19) of approximately 3.0 in nonvaccinated controls. The strains of the four species of chicken coccidia used were the Merck, Sharp and Dohme Research Laboratories strains of *E. tenella* (LS-18), *E. acervulina* (LS-3), *E. maxima* (FS-110) and *Eimeria necatrix* (AF1021).

Western blot (immunoblot) analysis. Sporulated oocysts from strains of all seven major species of chicken coccidia were obtained by standard procedures (13). The strains used in this analysis were *E. tenella* LS-18 and *E. necatrix* AF1021 and strains of *E. acervulina*, *E. maxima*, *Eimeria praecox*, *Eimeria mitis*, and *Eimeria brunetti* obtained from Peter Long, University of Georgia, Athens. (21, 30).

A crude antigen preparation was made from each of the strains by disrupting the oocysts fully by shaking them with glass beads (2-mm diameter) in Dulbecco's PBS (Ca^{2+} and Mg^{2+} free, pH 7.2) containing a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 mM 1,10-phenanthroline, 48 μg of soybean trypsin inhibitor per ml, 48 μg of aprotinin per ml, 0.04 mM leupeptin, 13 mM benzamidine, and 5 mM EDTA). Low-speed centrifugation (700 × *g*, 10 min) pellets were obtained from each disrupted oocyst preparation and were solubilized by heating them to 100°C for 5 to 10 min in sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.003% bromophenol blue in 62.5 mM Tris hydrochloride [pH 6.8]). Any particulate material was removed by centrifugation (13,000 × *g*, 5 min), and the protein content of the supernatant was determined by the modified Bradford method (38). The solubilized polypeptides of the seven samples were resolved by SDS-PAGE by using a Protean I chamber (Bio-Rad Laboratories) according to the manufacturer's instructions, which are based on the method of Laemmli (25). The samples, 50 μg of protein in sample buffer, were applied to a 10% polyacrylamide resolving gel with a 3% polyacrylamide stacking gel. Recombinant CheY-SO7′ (20 μg) and biotinylated SDS-PAGE molecular weight standards were also run in lanes adjacent to the oocyst samples. Following electrophoresis, the polypeptides were transferred to nitrocellulose (Hoefer Scientific Instruments) in a Transblot chamber (Bio-Rad Laboratories) according to the manufacturer's instructions, which are based on the method of Towbin et al. (41). The B antigens were localized in the transferred polypeptides by using a double antibody method (1) utilizing a Bio-Rad Immuno-Blot assay kit. Following incubation with the first antibody, a hyperimmune rabbit anti-CheY-SO7′ antiserum, the nitrocellulose sheet was incubated with the second antibody, a goat anti-rabbit immunoglobulin G (heavy and light)-alkaline phosphatase conjugate. B antigen localization was revealed with a color development substrate. The molecular weight standards were visualized by incubation with an avidin-alkaline phosphatase conjugate and then with the color development substrate (15).

Solid-phase fluorescence immunoassay. To determine whether chicken sera contained specific antibodies to either recombinant CheY-SO7′ or to whole sporulated oocyst antigen (SOA), a solid-phase fluorescence immunoassay was employed (44). Briefly, Immulon II 96-well microtiter plates (Dynatech Laboratories) were coated with antigen by incubating them with 100 μl of either B (CheY-SO7′) antigen or disrupted *E. tenella* SOA per well at protein concentrations of 10 μg/ml and 1 mg/ml, respectively, in coating buffer (0.02 M Tris buffer [pH 9.1] with 0.02% sodium azide). Plates were incubated at 37°C for 3 to 4 h, kept at 4°C overnight, washed with PBS containing 0.02% sodium azide (PBS/AZ), incubated with 200 μl of blocking buffer (1% bovine serum albumin [BSA] in PBS/AZ) per well for 2 h at 37°C, and washed with PBS/AZ. Various chicken serum samples (100 μl) diluted 1:50 with sample buffer (0.1% BSA in PBS/AZ)

were loaded into the wells and incubated for 2 h at 37°C. Following washes with PBS/AZ, phosphatase-conjugated goat anti-chicken immunoglobulin G (heavy and light) (Kirkegaard & Perry) at a concentration of 0.2 μg/ml in sample buffer was added to each well and incubated for 2 h at 37°C. After rinsing with Tris washing buffer (0.01 M Tris, 0.85% NaCl, 0.02% sodium azide), 100 μl of 0.01% 4-methylumbelliferyl phosphate in substrate buffer (50 mM sodium carbonate, 1 mM magnesium chloride [pH 9.8]) was added to each well, and the plate was incubated in the dark for 1 h at room temperature. Fluorescence intensity was quantified with a Titertek Fluoroscan microtiter plate reader (Flow Laboratories) with excitation and emission wavelengths of 455 and 480 nm, respectively.

RESULTS

Expression and purification of the CheY-SO7′ fusion protein. Figure 1A shows schematically the construction of the recombinant plasmid. Following transformation of the *E. coli* host, colonies were picked and the orientation of the SO7′ insert with respect to the CheY portion of the operon was determined by restriction enzyme mapping of the plasmid from each clone. The clone CheY-SO7′/9 had the correct orientation of SO7′ for the expression of a large fusion protein and was chosen for the purification of the recombinant antigen.

The fusion protein in the crude bacterial cell extract made from this clone, solubilized in guanidine HCl and urea, was applied to a hydroxyapatite column and was eluted with 10 and 20 mM sodium phosphate buffer washes. As shown in Fig. 1B, the clone CheY-SO7′/9 expressed a fusion protein with the appropriate molecular mass of approximately 36 kDa and was defined as a recombinant antigen of the B class on the basis of its reactivity with specific antisera (34a). Note that analysis of corresponding fractions from a bacterial lysate derived from a clone containing the CheY expression vector with SO7′ inserted in the reverse orientation did not reveal a protein of the characteristic size or immunoreactivity. For experimental vaccinations, purified recombinant antigen preparations were stored in buffer containing 7 M urea (22) at −20°C until use.

In vivo protection. Since its identification, several batches of the recombinant B antigen CheY-SO7′ have been produced and purified and have shown consistent protection in broiler chicks against coccidiosis caused by *E. tenella*, *E. acervulina*, and *E. maxima* infection (8). Over an extended period of time, several thousand broilers have been immunized with this antigen. The frequency distribution of lesion scores from large numbers of chicks provides a clear demonstration of the protective ability of CheY-SO7′ antigen. A comparison of the frequency distribution of lesion scores in immunized and nonimmunized broilers infected with *E. tenella* and *E. maxima*, pooled from several individual experiments involving several hundred chickens, is shown in Fig. 2A and B, respectively. In the nonimmunized group, >85% of the broilers infected with *E. tenella* developed lesion scores of ≥2.0 (mean lesion score = 2.90), while >80% of the broilers in the immunized group developed lesion scores of ≤2.0 (mean lesion score = 1.72). Similarly, in the nonimmunized group infected with *E. maxima*, >90% of the broilers developed lesion scores of ≥2.5 (mean lesion score = 3.04), while in the CheY-SO7′-immunized group, >85% of the broilers developed lesion scores of ≤2.5 (mean lesion score = 1.89). A similar frequency distribution of lesion scores was obtained from results with *E. acervulina* (results not shown).

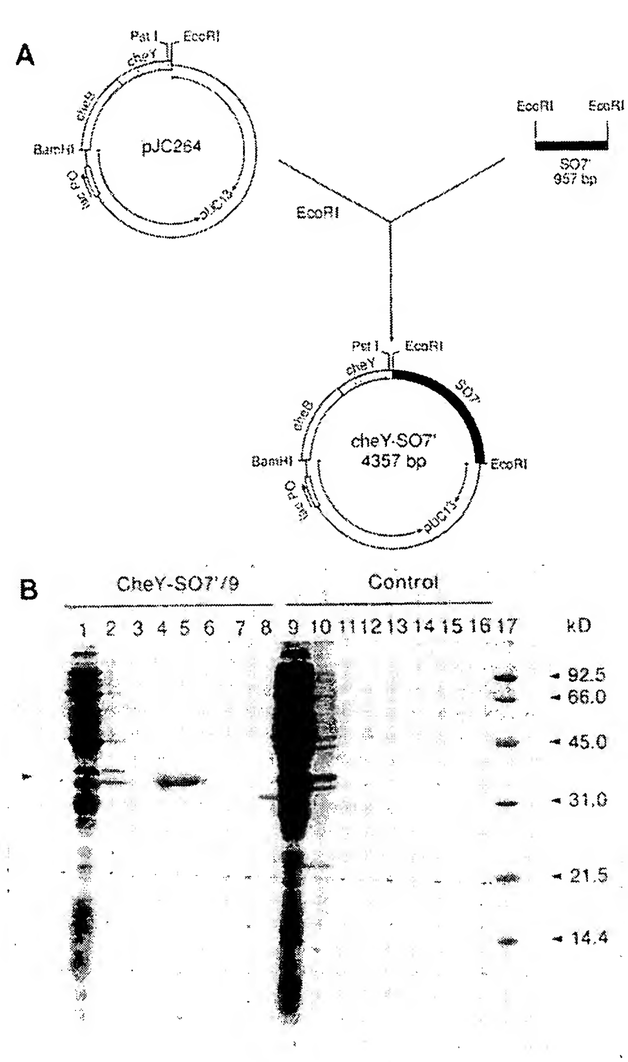

FIG. 1. Construction of plasmid CheY-SO7′ and expression of the CheY-SO7′ recombinant polypeptide antigen. (A) A cDNA fragment of 957 bp (28) containing a 216-amino-acid reading frame of the B antigen clone SO7′ was inserted, in frame, into the *Eco*RI site of the expression vector pJC264 (18). (B) SDS-polyacrylamide gel of fractions obtained during the purification procedure of the recombinant B antigen clone CheY-SO7′/9. Lanes 1 through 8: fractions from CheY-SO7′/9. The arrow on the left indicates the position of the recombinant B antigen. Lanes 9 through 16: fractions from a control clone containing the CheY expression vector with the SO7′ fragment inserted in the inverse orientation. Lanes 1 and 9: supernatant from whole bacterial lysate. Lanes 2 and 10: Triton X-100 wash of the insoluble pellet. Lanes 3 and 11: hydroxylapatite column flowthrough. The column was washed with 7 M urea containing sodium phosphate buffer (pH 6.5) at concentrations of 10 mM (lanes 4 and 12), 20 mM (lanes 5 and 13), 40 mM (lanes 6 and 14), 80 mM (lanes 7 and 15), and 160 mM (lanes 8 and 16). Lane 17: molecular weight standards.

In addition to protecting broilers against severe coccidiosis caused by *E. tenella*, *E. acervulina*, and *E. maxima*, the recombinant B antigen CheY-SO7′ also protects broilers against *E. necatrix*-induced coccidiosis (Fig. 3). The level of protection induced by CheY-SO7′ immunization is similar for all four species. However, unlike immunization by low-

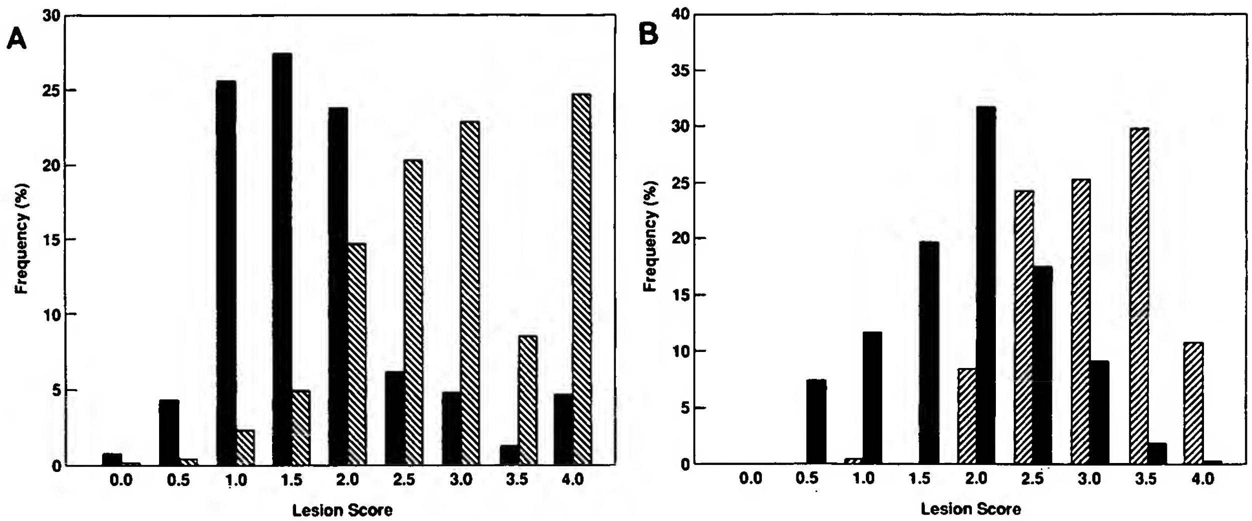

FIG. 2. Frequency distribution of lesion scores in 3- to 4-week-old chicks which were either immunized with various protective doses (0.1 to 10.0 μg) of recombinant B antigen (solid bars) or sham immunized with control buffer (hatched bars) and which were challenged with pathogenic doses of *E. tenella* (A) or *E. maxima* (B). A total of 626 broilers were immunized with CheY-SO7′ and challenged with *E. tenella*, yielding a mean lesion score of 1.72 (standard error = 0.02), and 317 broilers were sham immunized and challenged with the same dose of *E. tenella* oocysts, yielding a mean lesion score of 2.90 (standard error = 0.03). A total of 283 broilers were immunized with CheY-SO7′ and challenged with *E. maxima* oocysts, yielding a mean lesion score of 1.89 (standard error = 0.04), and 209 broilers were sham immunized and challenged with the same dose of *E. maxima* oocysts, yielding a mean lesion score of 3.04 (standard error = 0.04).

level infection (live vaccine groups), CheY-SO7′ immunization does not normally reduce the mean lesion score to below 1.5. As noted in Fig. 2, there tend to be a few chicks which do not respond to CheY-SO7′ immunization and subsequently develop severe coccidiosis when challenged with a pathogenic dose of sporulated oocysts. In contrast, the experimental groups immunized by live infection usually show a much tighter distribution of lesion scores and, by using an appropriate dose, individual lesion scores can be reduced to 1.0 or less (data not shown). Increasing the dose of CheY-SO7′ from 1 μg to 10 μg (Fig. 3) or increasing the number of doses (8) does not improve the observed protection.

Western blot analysis. CheY-SO7′ antigen was obtained from a cDNA library of *E. tenella* and has been shown to be present in the sporulated oocyst, localized in the refractile body of the sporozoite (34a). It is interesting that unlike live vaccination, which is species-specific, CheY-SO7′ cross-protects against at least four species of chicken coccidia (Fig. 3). Thus, to determine whether the B class of antigen is common to all species of chicken coccidia, immunoblots of SDS-PAGE-resolved polypeptides of sporulated oocysts of the seven species were undertaken. The localizing antibody preparation was a hyperimmune rabbit anti-CheY-SO7′ antiserum, and recombinant CheY-SO7′ antigen was used as the positive control. By using reactivity with the anti-CheY-SO7′ antiserum to define the B antigen class, all species of chicken coccidia were found to possess a predominant polypeptide band characteristic of B antigen (Fig. 4). As determined by the mobility of the molecular weight markers, the antigens from the various species have molecular masses of approximately 26 to 28 kDa, as shown previously for *E. tenella*, *E. acervulina*, and *E. maxima* (33). There is some interspecies variation in the relative mobilities of the B antigens, which may reflect differences in the molecular weight and/or other characteristics of the antigen between the different species of coccidia. As expected, the recombinant B antigen CheY-SO7′ has a higher molecular mass (approximately 36 kDa) because of the contribution of CheY in the fusion protein (22).

Chicken antibody to recombinant CheY-SO7′. It is known that eimerian sporozoites are very sensitive to the action of antibody and complement (5) and that chickens can be partially protected from coccidiosis by passive transfer of specific antibody (6, 36, 43). To ascertain whether the CheY-SO7′ immunization of broiler chicks induced antibody production, serum samples were obtained from chicks which had undergone a variety of immunization regimens. Since the observed protection was induced by purified antigen without any adjuvant, no adjuvant was used in any of these immunizations.

Young chicks, less than 14 days old, which had not been immunized with CheY-SO7′ had demonstrable levels of antibody to both CheY-SO7′ and the crude parasite extract, SOA (Table 1). Since these chicks were very young and were reared in isolation, it can be assumed that this reactivity is due to maternal antibody; sera from chicks older than two weeks of age did not demonstrate this reactivity. Immunization with CheY-SO7′ did not induce specific antibody in either young or older chicks. The only other chicks tested which demonstrated CheY-SO7′-specific antibody in their sera were those hyperimmunized with whole sporozoite antigen in Freund's adjuvant and the much older chicks from breeder flocks which had been exposed to live coccidia for extended periods of time.

DISCUSSION

Problems incurred because of the emergence of drug-resistant strains of *Eimeria* spp. and the difficulty in discovering and developing new anticoccidial agents have emphasized the need for novel approaches for the control of coccidiosis in the poultry industry (4). It is well established that infection with virulent (14, 42) or attenuated (20) strains

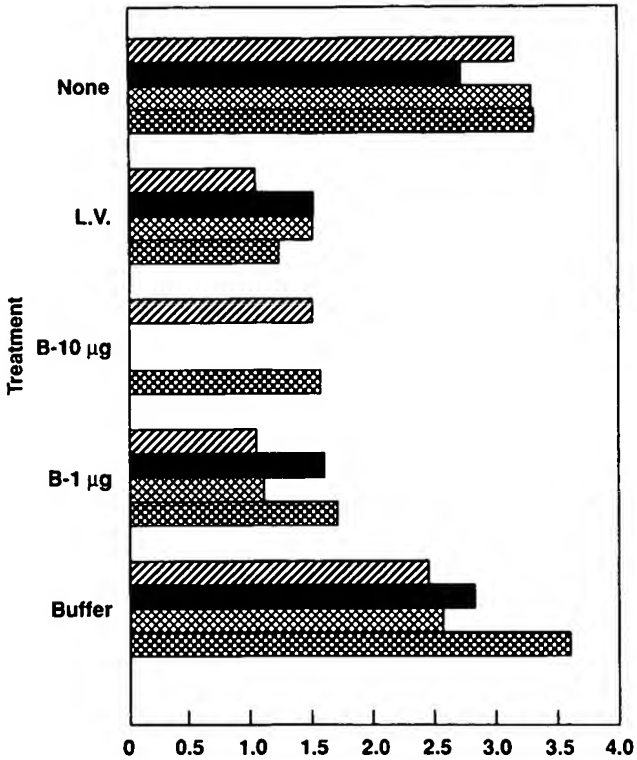

FIG. 3. Recombinant B antigen-induced cross protection of 3- to 4-week-old broilers against *E. tenella* ( ), *E. acervulina* ( ), *E. maxima* ( ), and *E. necatrix* ( ). Groups (8 to 10 chicks per group) of 2- to 4-day-old chicks either were immunized with 1 µg (B-1 µg) or 10 µg (B-10 µg) of B (CheY-SO7′) antigen per chick, were sham immunized with buffer (Buffer), were inoculated with an immunizing dose of sporulated oocysts equivalent to a live vaccine (L.V.), or were untreated (None). The live vaccine dose was 100 sporulated oocysts per chick for *E. tenella*, 2,000 sporulated oocysts per chick for *E. acervulina*, 50 sporulated oocysts per chick for *E. maxima*, and 1,000 sporulated oocysts per chick for *E. necatrix*. At approximately 3 weeks of age, the chicks were challenged with pathogenic doses of sporulated oocysts of one of the four species tested, and lesion scores were evaluated 6 days postinfection for *E. acervulina* and 7 days postinfection for *E. tenella*, *E. maxima*, and *E. necatrix*. The bars represent mean lesion scores; standard errors of the mean ranged between 0.08 and 0.29.

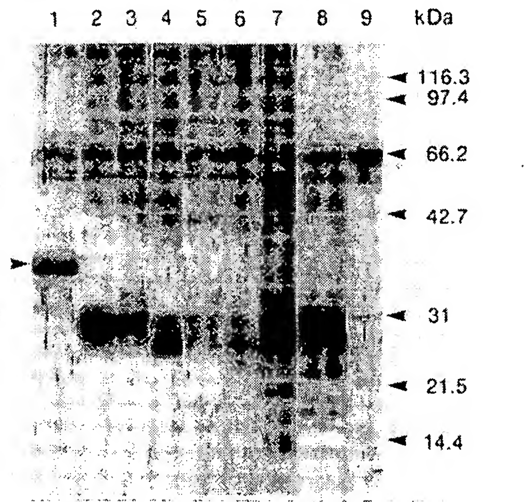

FIG. 4. Western blot analysis of sporulated oocyst preparations from all seven major species of chicken coccidia. The oocyst preparations (lanes 2 through 8), purified CheY-SO7′ (lane 1, arrow), and biotinylated molecular weight markers (lane 9) were resolved by SDS-PAGE, transferred to a nitrocellulose sheet which was incubated sequentially with hyperimmune rabbit anti-CheY-SO7′ antiserum, a goat anti-rabbit immunoglobulin G (heavy and light)-alkaline phosphatase conjugate, and the color development substrate. The molecular weight markers were visualized by incubation with an avidin-alkaline phosphatase conjugate and then with the color development substrate. Lanes: 2, *E. necatrix*; 3, *E. tenella*; 4, *E. acervulina*; 5, *E. maxima*; 6, *E. praecox*; 7, *E. brunetti*; 8, *E. mitis*.

of coccidia induces strong, species-specific, protective immunity in chickens. Nevertheless, the use of available live vaccines consisting of virulent strains for the control of coccidiosis is not widespread but is limited mostly to chicken breeder flocks, suggesting that such vaccines are not suitable for broiler production. Attenuated live vaccines for the broiler industry are being developed (3, 16, 34). However, as with other live vaccines, there are concerns about stability, quality control, cost-effectiveness, and efficacy against the vast array of field strains likely to be encountered in different geographical areas (4, 9). The development of a subunit vaccine would circumvent some of these problems.

The recombinant antigen (CheY-SO7′) described here is remarkable in that it induces cross-protective immunity against at least four major species of coccidia and is effective at relatively low doses (8). On the basis of the immunoblot analysis which demonstrates the presence of B antigens in all seven species, it is possible that a single recombinant antigen could cross-protect against not only all chicken coccidia but also against other species. However, in contrast to live vaccination, B antigen, even at relatively high doses, does not induce sterile immunity nor does it fully prevent disease. These results indicate some fundamental differences between immunity induced by mild infection and that induced by the B antigen; it would appear that B antigen is not

TABLE 1. Reactivity of chicken sera with recombinant B (CheY-SO7′) antigen and *E. tenella* SOA[a]

Serum type	Antibody-antigen reactivity (fluorescence units)	
	CheY-SO7′	SOA
Preimmune from 2-day-old chicks	6,393	7,571
Preimmune from 2-wk-old chicks	1,570	1,343
Nonimmunized from 4-wk-old chicks	1,128	1,222
CheY-SO7′-immunized from 4-wk-old chicks[b]	872	628
Hyperimmune chick anti-sporozoite serum[c]	9,701	>10,000[d]
Breeder chick[e]	7,788	9,750

[a] Reactivity determined in a solid-phase fluorescence immunoassay.
[b] Chicks were immunized intramuscularly with 1 µg of CheY-SO7′ antigen 2 days, 2, 9, and 16 days, or 16, 23, and 30 days after hatching, and serum was taken at weekly intervals up to 7 weeks of age. These results are examples of several samples tested, none of which demonstrated positive reactivity.
[c] Serum from chicks immunized with disrupted sporozoites in Freund's complete adjuvant and then with sporozoite antigen in Freund's incomplete adjuvant at regular intervals until serum became hyperimmune (5).
[d] Fluorescence signal was beyond the upper threshold of the microtiter plate reader.
[e] Chick serum samples obtained from Spafas Inc., Storrs, Conn.

involved in establishing species-specific immunity effected by live infection.

As expected with the low antigen doses used in the absence of any adjuvant, B antigen immunization did not elicit antibody production, indicating that cell-mediated mechanisms may be involved in the observed partial protection. However, it is interesting to note that chicks exposed to parasite antigens either through prolonged, natural exposure (as with breeder chicks) or by hyperimmunization do produce antibodies to B antigen. Since B antigen immunity is incomplete (that is, it provides partial protection only), either enhanced antigen presentation, such as with a viral vector, or the addition of other antigens should improve efficacy to a level at which such a vaccine (subunit) would be competitive with chemotherapy.

Contained within the sequence of SO7′ (28) and at its carboxy terminus is the sequence of another partially protective, recombinant antigen, GX3262 (31). However, there are major differences between the abilities of the two antigens to immunize chicks against coccidiosis. In contrast to GX3262, a single low dose (10 ng) of CheY-SO7′ in the absence of any adjuvant is sufficient to protect 2-day-old chicks against severe coccidiosis caused by infection with any one of several species of coccidia for several weeks after immunization (8). Whether this is due to the larger size of SO7′, providing more, important epitopes (at the amino terminus), or due to the fact that CheY-SO7′ is a CheY fusion protein (rather than a β-galactosidase fusion protein) is under investigation. The ability to obtain CheY fusion protein with high purity (22) may also contribute to the low-dose efficacy (based on total protein per dose) of CheY-SO7′. These differences also exist between CheY-SO7′ and other refractile body recombinant antigens (11). Nevertheless, despite qualitative and quantitative differences, it is interesting that several protective antigens have been identified which originate in the refractile body of the parasite.

All of the studies reported here have been conducted in our cage model for chicken coccidiosis. In addition to studies aimed at improving the efficacy of the B antigen so that mean lesion scores would be reduced to below 1.0 in immunized chicks, other studies to demonstrate protection against coccidiosis, including productivity competitive with chemotherapy under floor-pen conditions, are ongoing.

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EXHIBIT D

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Tandem trinucleotide repeats throughout the nucleotide sequence of a cDNA encoding an *Eimeria tenella* sporozoite antigen

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Submitted July 31, 1989 EMBL accession no. X15898

Polyclonal anti-*Eimeria tenella* sporozoite antibody was used to isolate cDNA clone S07 from a λgt11 expression library constructed using mRNA from *E. tenella* sporulating oocysts[1]. The nucleotide sequence of clone S07 was determined by dideoxy sequencing of ExoIII generated deletion mutants[2] from both ends of the cDNA in pUC119 and is shown here. The sequence is 957 nucleotides long and the deduced amino acid sequence predicts the occurrence of only one in-frame methionine residue at nucleotide position 65. This ATG codon is in an environment that is favorable for translation initiation by eukaryotic ribosomes[3]. Initiation at this position would result in an open reading frame of 216 amino acids with a predicted molecular weight of 22.4 kD. A striking feature of the sequence is the tremendously high frequency of the trinucleotide AGC dispersed in a tandemly repeated fashion throughout the length of the clone, i.e., in both protein coding and untranslated regions. Interestingly, those trinucleotide repeats located within the protein coding region are not in the same reading frame. The majority code for either serine (AGC) or glutamine (CAG) residues. In both of these, the third position is a G or C nucleotide which is in good agreement with the overall 69.5% G/C content for this cDNA and the 83% third position G/C bias within the open reading frame.

```
1     CTC GCC CCA ACT TTT TCC CCC GCG CTC CGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AAA ATG GCA GAC CTC TTC AGC GGA CTC GTG GGC GGC GTC GTC GGC GCT  108
                                                                                       M   A   D   L   F   S   G   L   V   G   G   V   V   G   A

109   GTT GCT GCA GCA GAT TTG CCT GCG GAG GGC GAG AGG GCC CCC CGC CCC GCC CCC GGC ACT GCC TGG ACT TGC TGC TGC AGC AAA CTG CAA GAA GGG GCC CGC GAG CTG  216
       V   A   A   A   D   L   P   A   E   G   E   R   A   P   R   P   A   P   G   T   A   W   T   C   C   C   S   K   L   Q   E   G   A   R   E   L

217   GAG GGT TTT GTG CAG CAG CTG AGT TTT GTT GCA GGG AAG CTG GCC TGC TGC CTG CGG GTG GGG GCG GAG CAG CTG GCG CGC TGC GCT GCG GAG GGG CGG CTG CCC AGC  324
       E   G   F   V   Q   Q   L   S   F   V   A   G   K   L   A   C   C   L   R   V   G   A   E   Q   L   A   R   C   A   A   E   G   R   L   P   S

325   AGC AGC AGC AGC AGC AGC TGC TGC GCG CTG CTG CAG CTC GAG AAG CAG GAC CTC GAG CAG AGC CTC GAG GCC GGC AAG CAG GGC GCG GAG TGC CTC TTG AGG AGC AGC  432
       S   S   S   S   S   S   C   C   A   L   L   Q   L   E   K   Q   D   L   E   Q   S   L   E   A   G   K   Q   G   A   E   C   L   L   R   S   S

433   AAA CTG GCC CTC GAG GCC CTC CTC GAG GGG GCC CGC GTT GCA GCA ACG CGG GGT TTG CTG CTG GTC GAG AGC AGC AAA GAC ACG GTG CTG CGC AGC ATT CCC CAC ACC  540
       K   L   A   L   E   A   L   L   E   G   A   R   V   A   A   T   R   G   L   L   L   V   E   S   S   K   D   T   V   L   R   S   I   P   H   T

541   CAG GAG AAG CTG GCC CAG GCC TAC AGT TCT TTC CTG CGG GGC TAC CAG GGG GCA GCA GCG GGG AGG TCT CTG GGC TAC GGG GCC CCT GCT GCT GCT TAC GGC CAG CAG  648
       Q   E   K   L   A   Q   A   Y   S   S   F   L   R   G   Y   Q   G   A   A   A   G   R   S   L   G   Y   G   A   P   A   A   A   Y   G   Q   Q

649   CAG CAG CCC AGC AGC TAC GGG GCG CCC CCC GCC TCC AGC CAG CAG CCC TCC GGC TTC TTC TGG TAG CCC TGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC GCG  756
       Q   Q   P   S   S   Y   G   A   P   P   A   S   S   Q   Q   P   S   G   F   F   W   *

757   GGC GGC AGC CGC GGC GGG GCC GGG GCG CCG CTG CAG CAA CAG CAG CAG CCG GCC CGG CTA GCG CCG CGG AGC ACT CGC AGG GAA CTC CAC AGG CAG CGG GAG AGC AGC  864

865   AGG GAC GAG AAG CAG GTC ATG TAG CGC AGG CAG CAG CGC CAG CTG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CTC CTG CAC CG
```

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☐ **1:** 84175. Reports ...[gi:84175] This record has been discontinued.

Comment Features Sequence

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ACCESSION   S05575
VERSION     S05575  GI:84175
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            summary: #length 216 #molecular-weight 22369 #checksum 3651
            ;
            PIR dates: 07-Sep-1990 #sequence_revision 07-Sep-1990 #text_change
            18-Jun-1993
            .
KEYWORDS    .
SOURCE      Eimeria tenella
  ORGANISM  Eimeria tenella
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eimeriida; Eimeriidae;
            Eimeria.
REFERENCE   1  (residues 1 to 216)
  AUTHORS   Liberator,P.A., Hsu,J. and Turner,M.J.
  TITLE     Tandem trinucleotide repeats throughout the nucleotide sequence of
            a cDNA encoding an Eimeria tenella sporozoite antigen
  JOURNAL   Nucleic Acids Res. 17 (17), 7104 (1989)
   PUBMED   2780319
COMMENT     [WARNING] On Oct 25, 2005 this sequence was replaced by a newer
            version gi:113933.
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                     /db_xref="taxon:5802"
     Protein         1..216
                     /product="sporozoite antigen"
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       61 vagklacclr vgaeqlarca aegrlpssss ssccallql ekqdleqsle agkqgaecll
      121 rssklaleal legarvaatr glllvesskd tvlrsiphtq eklaqayssf lrgyqgaaag
      181 rslgygapaa aygqqqqpss ygappassqq psgffw
//
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